IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	
APPLICANT: Okunji, et al.	DATE:
SERIAL NO.: 09/428,203	GROUP ART UNIT: 1651
FILED: October 27, 1999	EXAMINER: M. Flood
FOR: PLANT DERIVED ANTI-PARASITIC AND ANTI-FUNGAL COMPOUNDS AND METHODS FOR EXTRACTING THE COMPOUNDS	**09/428,203 (Okunji, et al.) Attachment A (14 Pages)**

RULE 132 DECLARATION

1. I, Christopher O. Okunji, do hereby declare that:

2. I am a Ph.D. Pharmacognosist, Senior Research Associate at LTS Corporation, Bethesda, Maryland.

During the patent application process my employment as Senior PhD Research Pharmocognosist, was as follows:

Date	Patent Information activities	Employment (agency paid salary)
1990-1993	invention conceived	University of Nigeria, Nsukka
03-1993-1996	invention developed first drafted	National Science Foundation and International cooperative Biodiversity Grant (ICBG), USA
1996-	invention disclosed (provisional)	ICBG - fund from NIH
	invention submitted as PTO application	ICBG - fund from NIH
06-03-2005	invention prosecution	ICBG (left WRAIR)
06-04-2004 10-23-2005	"	self-employed
10-24-2005 04-28-2006	"	Rutgers University, Biotech Center, NJ
05-01-2006 – present	"	LTC Corporation (Fed contractor)

3. I wish to state that the references of record by researchers/scientists (Mbah, et al., and Ekpendu, et al.) were made known to me, and that my work as stated in the above-identified patent application is substantially different from the art of record. My work on *Napoleonaea* is part of my Ph.D. dissertation, specifically the use of seeds vs. that of other plant parts such as extracts. (see detail item 6). To illustrate the above points, my research interest on the genus *Napoleonaea* started as far back as 1983 when I screened this plant for molluscicidal activity. My Ph.D. dissertation in 1987, entitled "Molluscicidal and Antifungal Properties of Some Nigerian Medicinal Plants,"
http://www.unn-edu.net/postgrad/pg fac of pharm sci.htm
identified *Napoleonaea vogeli* auct. (Fam. Lecythidaceae; synonyms: *Napoleonaea imperialis* P. Beauv.) as potential plant molluscicides. Further screening of this plant for antileishmanial activity in 1993 identified *Napoleonaea imperialis* seeds as having a promising antileishmanial activity. Bioassay guided-chromatographic fractionation of *Napoleonaea imperialis* seeds yielded imperialisides (A-C).

4. That the work of Mbah, et al., was on the antibacterial activity of extracts from leaves, stem-bark, root and root-bark

of *Napoleonaea imperialis* without specific identification of any phytochemical constituent, while my work on the seed specifically identified three antileishmanial compounds belonging to the class saponin. Please note that the conclusion of the Mbah, et al., reference states: "...the absence of toxic affects for the flavonoids and triterpenoids is very important for testing their eventual (emphasis added) activity on human lymphocytes." No conclusive data on human activity had been established. Thus, the use of ethanol in extraction is insignificant to Dr. Mbah, et al.

5. Similarly, Dr. Ekpendu's group studied "hexane, ethyl acetate and methanol extracts of Napoleonaea imperialis, obtained from the root bark, not the extracts from the seeds themselves." As established in my patent application, the seeds were chosen by my group to obtain biologically active extracts showing antileishmanial activity.

6. A critical review of the referenced publications on *Napoleonaea imperialis* P.Beauv(Lecythidaceae) by Ekpendu *et al* (1998) and Kapundu *et al* (1980) revealed the following observations; first, the two groups of investigators were chemists and therefore were more interested on the chemistry of this plant rather than their biological or therapeutic

properties. Secondly, neither Ekpendu nor Kapundu screened for biological or pharmacological activities of the constituents of this plant. Also both groups used similar methods in their chemical investigation of the major constituents of *N. imperialis* known as saponins. In all, both referenced papers the saponins were first hydrolyzed before isolation and chemical identification of the constituents. It is remarkable to note that both groups worked on the hydrolyzed products (sapongenols/sapogenins/aglycones/genin) instead of the intact plant constituents (saponins). The implication of these approaches will be discussed in details. Some investigators adopted hydrolysis method because it eliminates the sugars resulting to simpler compounds. The product of hydrolysis is simpler, yielding lower molecular weight compounds, less polar, less complex structurally and easy to handle.

In contracts, as a pharmacognosist, I was particularly interested in the un-hydrolyzed, naturally occurring and pharmacological/ biological active plant constituents. My approach eliminated all processes of hydrolysis for compounds submitted for biological testing. In fact only naturally occurring pharmacologically active compounds were pursued further rather than hydrolyzed products. Bioassay directed-chromatographic fractionations of active extracts were

undertaken leading to the isolation of naturally occurring saponins hereby eliminating art fats.

To fully appreciate the distinction made above between the approaches adopted by Ekpendu and Kapundu while investigating *N. imperialis* seeds and root-bark respectively and that of mine investigating the seeds of *N. imperialis*, it is important to examine the constituents of this plant. Our present knowledge on *N. imperialis* indicated that the major constituents of this plant are the saponins, although there is very scanty information available in the literature on this plant.

Saponins are high-molecular-weight glycosides, consisting of a **sugar moiety** linked to a triterpene or steroid (**aglycone**).

Therefore, Saponin = Sugar + Aglycone (triterpene or steroid).

All saponins have in common the attachment of one or more sugars to the aglycone. Saponins are extremely widely distributed in the plant kingdom. Saponins occur in some plants which are used as human food. The list of biological activity associated with saponins is very long.

Saponin contents of different morphological plant part:

Plant secondary metabolites such as saponins, alkaloids, flavonoids etc have been report to vary in their distribution in different plant parts. In these examples, saponin contents have been reported to vary depending on factors such as the cultivar, the age, the physiological state and the geographical location of the plant (Hostettmann and Maraton, (1994)). Considerable variation in composition and quantity of saponins in vegetable material from different places, as documented for *Lonicera japonica* (Caprifoliaceace) has been reported (Kawai *et al*, 1998).

The saponin distribution among the organs of a plant may vary considerably. In the garden marigold (*Calendula officinalis*, Asteraceae), for example saponins with a glucuronic acid moiety at C-3 of oleanolic acid are founding the flowers, while a glucose moiety at the same position is found in the saponins from the roots (Lutomski, 1983; Vidal-Ollivier et al. 1989a,b). The flowers contain 3.57% saponins, while the roots have 2.55% of their dry weight in the form of saponins (Isaac, 1992). Ginsenoside levels in Panax ginseng (Araliaceae) are lowest in the leafstalks and stem (0.77%), intermediate in the main root (1.3%) and lateral roots (3.5%) and highest in the leaves (5.2%) and root hairs (6.1%) (Koizumi *et al*. 1982).

The above examples address some of the examiner's concerns with regards to the composition of different parts of the plant parts; root-barks vs seeds. Our work on Dracaena species revealed that very high saponin content are found mostly in the seeds (Okunji *et al* 1996)

Problems Associate with Hydrolysis: Method adopted by Ekpendu and Kapundu

Numerous chemical reactions and methods have been employed for breaking down saponins into smaller units for more ready analysis (Kitagawa 1981), one of such method is acid hydrolysis.

It is believed that once acid hydrolysis is completed, then the aglycone will be separated and identified. Many chemists including Ekpendu *et al* (1998) and Kapundu *et al* (1980) adopted hydrolysis of saponins prior to chemical characterization of plant constituents. However, there are some significant concerns such as artifacts formation, not being able to obtain genuine aglycone, possibility of epimerization, transformation etc. The following paragraphs will illustrate the above pitfalls in detail.

It has been reported that acid hydrolysis is not without risk because prolonged heating with an inorganic acid can give rise to complications involving artifact formation, low yields

and low selectivity (Tschesche and Wulff, 1972; Kitagawa, 1981). This is true not only of triterpene saponins but also of steroid saponins and saponins from marine organisms (Kitagawa *et al.* 1985b), often making the job of structure elucidation very complicated. A typical example from a study of the effects of various hydrolytic procedure on the sapogenin profile of soya saponins has shown that soyasapogenols B_1, C, D and E are probably formed as artifacts on aqueous hydrolysis of soya flour with hydrochloric acid in ethanol (Ireland *et al.* 1987). The true aglycones, soyasapogenols A and B are obtained by hydrolysis with sulphuric or hydrochloric acid in anhydrous methanol (Ireland and Dziedzic, 1986). Another problem arises during the acid hydrolysis of oleanolic acid and hederagenin glycosides in dioxin, giving rise to possible formation of lactone (Hiller *et al.* 1987). Similarly, Sulphuric acid on hydrolysis of hovenosides (glycosides of jujubogenin) gave a lactone, ebelin lactone (Inoue *et al.* 1978).

It is sometimes very difficult to obtain the genuine aglycones from the parent saponins. This problem is especially acute for the triterpenes containing a 13β, 28-oxide structure. Tscheshe and coworkers stated that it took a long time, for example, before the aglycone cyclamiretin A of cyclamen (from the tubers of *Cyclamen europaeum,* Primulaceae) was completely characterized (Tscheshe *et al.* 1966). Another example is the

case for other 13β, 28-oxide aglycones (protoprimulagenin A), saikogenin F, etc.), it could be easily ring-opened by acid to the corresponding 12-en-28-alcohol. Similarly Primulaginin A is most probably an artifact produced during hydrolysis of saponins containing protoprimulagenin A as aglycone (Tscheshe *et al.* 1983). It should be noted here, however, that not all 12-en-28-alcohol aglycones are artifacts.

On hydrolysis, an acid-catalysed double-bond migration in triterpenes can also occur. For example, some olean-12-enes are isomerized to olean-13(18)-enes with hydrochloric acid in aqueous ethanol (Kubota *et al.* 1969). Quallaic and echinocystic acids are both isomerized to the corresponding olean-13(18)-enes under these conditions (Kubota *et al* 1969).

Epimerization is possible during acid hydrolysis, as shown by the conversion of arjungenin (from the corresponding 28-glycoside) to tomentosic acid. This transformation proceeds through the pathway described by Mahato *et al.* 1990 and confirmed by the isolation of the intermediate lactone.

The transformation of cochalic acid to echinocystic acid is another example. It involves an epimerization at the 16-OH group and probably also occurs via a 28 → 16 lactone (Mahota *et al.* 1990).

These are very few examples of potential risk of isolating art facts instead of naturally occurring plant constituents

associated when adopting acid hydrolysis in duringphytochemical investigations. It is possible that some of the Ekpendu's compounds were artifacts. The compounds isolated by my method have been replicated several times.

In conclusion; as reported Ekpendu *et al* (1998), hydrolysis of both ethyl acetate and methanol extracts were undertaken leading to the isolation of series of less polar compounds when compared with unhydrolyzed compounds. Similarly, control hydrolysis was carried out by Kapundu *et al* (1980) leading to characterization of new "prosapogenins" napoleogenol and napoleogenin which were less polar relative to un- hydrolized counterpart.

The approach adopted by both Ekpendu *et al* (1998) and Kapundu *et al* (1980) differ significantly from mine in that in my investigation, no acid hydrolysis was used instead intact saponins were isolated and characterized. In addition biological testing was carried out leading to the identification of bioactive compounds. The distribution of secondary metabolites in different plant parts has been discussed above. The results showed that different constituents have been isolated from different plant parts.

7. These facts would have been corroborated by Dr. Ekpendu, who is known to me. However, due to extreme hardships and all attempts to contact Dr. Ekpendu failed even during my several trips to Nigeria between 2000-2003. These hardships included economic and financial constraints, lack of communication or national database or national phonebook etc. Additionally several attempts to contact students (who had worked on the experiments leading to Dr. Ekpendu's results) also failed. Due to the lack of proper communication facilities and the absence of demographic databases, any information on these students were unattainable since they had subsequently graduated.

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I further declare under penalty of perjury, pursuant to the laws of the United States of America, that the foregoing is true and correct, and that this declaration was executed by me on December 19, 2006, at Silver Spring Maryland.

Christopher O. Okunji 12/19/2006